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Aminopyridines Potentiate Synaptic and Neuromuscular Transmission by Targeting the Voltage-activated Calcium Channel β Subunit*◆

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Aminopyridines such as 4-aminopyridine (4-AP) are widely used as voltage-activated K^+ (Kv) channel blockers and can improve neuromuscular function in patients with spinal cord injury, myasthenia gravis, or multiple sclerosis. Here, we present novel evidence that 4-AP and several of its analogs directly stimulate high voltage-activated Ca^{2+} channels (HVACCs) in acutely dissociated neurons. 4-AP, 4-(aminomethyl)pyridine, 4-(methylamino)pyridine, and 4-di(methylamino)pyridine profoundly increased HVACC, but not T-type, currents in dissociated neurons from the rat dorsal root ganglion, superior cervical ganglion, and hippocampus. The widely used Kv channel blockers, including tetraethylammonium, α-dendrotoxin, phrixotoxin-2, and BDS-I, did not mimic or alter the effect of 4-AP on HVACCs. In HEK293 cells expressing various combinations of N-type (Cav2.2) channel subunits, 4-AP potentiated Ca^{2+} currents primarily through the intracellular β_3 subunit. In contrast, 4-AP had no effect on Cav3.2 channels expressed in HEK293 cells. Furthermore, blocking Kv channels did not mimic or change the potentiating effects of 4-AP on neurotransmitter release from sensory and motor nerve terminals. Thus, our findings challenge the conventional view that 4-AP facilitates synaptic and neuromuscular transmission by blocking Kv channels. Aminopyridines can directly target presynaptic HVACCs to potentiate neurotransmitter release independent of Kv channels.

Aminopyridines such as 4-aminopyridine (4-AP)[3] and 3,4-diaminopyridine (3,4-DAP) are widely used as voltage-activated K^+ (Kv) channel blockers and can improve neuromuscular function in patients with multiple sclerosis (1), spinal cord injury (2), myasthenia gravis (3), or Lambert-Eaton syndrome (4, 5). The classical view is that the beneficial effect of 4-AP results from blocking Kv channels, which leads to increases in the duration of action potentials, Ca^{2+} influx, and neurotransmitter release (6–8). This conception assumes that voltage-activated Ca^{2+} channels (VACCs) are stimulated indirectly by increased excitability of cells after Kv channels are blocked by 4-AP. However, other Kv channel blockers such as tetraethylammonium (TEA) have limited effects in potentiating neurotransmitter release and improving neuromuscular function. Furthermore, 4-AP is effective in the treatment of patients with VACC antagonist overdose (9), Lambert-Eaton syndrome caused by impairment of presynaptic VACCs (10), or episodic ataxia type 2, a disorder caused by mutation of Cav2.1 (11). These observations raise the possibility that 4-AP may directly stimulate VACCs in addition to its effect on Kv channels.

Although VACCs are essential for synaptic and neuromuscular transmission, there is no evidence that 4-AP can directly stimulate VACCs in neurons. In this study, we investigated the direct effect of 4-AP on VACCs in dissociated neurons and its role in potentiating neurotransmitter release. We discovered that 4-AP and several of its analogs have a profound potentiating effect on high voltage-activated Ca^{2+} channels (HVACCs) independent of Kv channels. The intracellular β subunit is largely responsible for this potentiating effect. Furthermore, 4-AP potentiates neurotransmitter release from both sensory and motor nerve terminals independent of Kv channels. Our findings strongly suggest that aminopyridines facilitate synaptic and neuromuscular transmission primarily through direct stimulation of HVACCs. 4-AP and 3,4-DAP have been used as specific Kv channel blockers for >30 years, and their direct effects on HVACCs have long been overlooked. The interpretation of their effects on the intracellular Ca^{2+} level and neurotransmitter release should be revised on the basis of this new information.

EXPERIMENTAL PROCEDURES

Isolation of Dorsal Root Ganglion, Superior Cervical Ganglion, and Hippocampal Neurons—Male Sprague-Dawley rats (5–6 weeks old; Harlan, Indianapolis, IN) were anesthetized with isoflurane and then rapidly decapitated. The thoracic and lumbar segments of the vertebral column were dissected. The dorsal root ganglions (DRGs) were quickly removed and transferred immediately into Dulbecco's modified Eagle's medium (DMEM; Invitrogen). The ganglion fragments were placed in a flask containing 5 ml of DMEM in which trypsin (type I, 0.5 mg/ml; Sigma) and collagenase D (1 mg/ml; Pfizer) had been

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[3] The abbreviations used are: 4-AP, 4-aminopyridine; 3,4-DAP, 3,4-diaminopyridine; Kv, voltage-activated K^+; VACC, voltage-activated calcium channel; TEA, tetraethylammonium; HVACC, high voltage-activated calcium channel; DRG, dorsal root ganglion; DMEM, Dulbecco's modified Eagle's medium; SCG, superior cervical ganglion; EPSC, excitatory postsynaptic current; EPP, end-plate potential.

dissolved. After incubation at 34 °C in a shaking water bath for 30 min, soybean trypsin inhibitor (type II-s, 1.25 mg/ml; Sigma) was added to stop trypsin digestion. The cell suspension was subjected to centrifugation (500 rpm, 5 min) to remove the supernatant and replenished with DMEM. The cultured DRG neurons were replenished with DMEM containing 10% fetal bovine serum (Invitrogen) and penicillin/streptomycin/glutamine supplement (1%; Invitrogen). Neurons were then plated onto a 35-mm culture dish containing poly-L-lysine (50 μg/ml)-precoated coverslips and kept for at least 1 h before electrophysiological recordings (12).

The superior cervical ganglion (SCG) neurons were removed and placed in cold (4 °C) DMEM. The ganglion fragments were then placed in a flask containing 5 ml of DMEM in which trypsin and collagenase D had been dissolved. After incubation at 34 °C in a shaking water bath for 30 min, the flask was shaken vigorously by hand to release the neuronal somata from the fragments. Soybean trypsin inhibitor was then added to stop trypsin digestion. The cell suspension was subjected to centrifugation (500 rpm, 5 min) to remove the supernatant and replenished with DMEM.

To dissociate hippocampal neurons, neonatal rats (2–3 days old) were deeply anesthetized with isoflurane and then decapitated. The whole brain was removed and placed immediately in ice-cold DMEM. The hippocampus was identified and dissected under a microscope, and neurons were isolated by an enzymatic protocol. The tissues were incubated in DMEM with 0.25% trypsin for 15 min at 37 °C, and neurons were mechanically dissociated using a glass pipette. The neurons were then plated (1.5×10^6 cell/ml) on poly-L-lysine-precoated coverslips. The isolated neurons were grown in DMEM containing 10% fetal bovine serum and penicillin/streptomycin/glutamine supplement for 24 h. The neuron basal medium (2% B-27; Invitrogen) was replaced every 3–4 days.

Cell Culture and Transfection—Human embryonic kidney HEK293 cells were grown in DMEM (containing 10% fetal bovine serum but no antibiotics) to 80% confluence and maintained at 37 °C in a humidified incubator with 5% CO_2. The cells were transiently transfected with N-type Ca^{2+} channel subunits, including α_1B (from rat SCG), $\alpha_2\delta$ (from rat SCG), and β_3 (from rat brain), or Cav3.2α cDNA (from rat brain), all in pcDNA3.1 using Lipofectamine 2000 (Invitrogen). When expression of more than one subunit was induced, the multiplasmids were cotransfected at a 1:1 ratio. Fifteen hours after transfection, the cells were dissociated with 0.05% trypsin. Cells were then plated onto a 35-mm culture dish containing poly-L-lysine-precoated coverslips and kept for another 18 h in an incubator at 30 °C before electrophysiological recordings.

Electrophysiological Recordings—Electrodes with a resistance of ~2 megaohms were pulled from glass capillaries using a micropipette puller and fire-polished. Neurons were recorded in the whole-cell configuration using an EPC-10 amplifier (HEKA Instruments, Lambrecht, Germany). After whole-cell configuration was established, the cell membrane capacitance and series resistance were electronically compensated. All experiments were performed at room temperature (~25 °C). Signals were filtered at 1 kHz, digitized at 10 kHz, and acquired using the Pulse program (HEKA Instruments). The whole-cell Ca^{2+} current, carried by barium (I_{Ba}), was recorded using an extracellular solution consisting of 140 mM *N*-methyl-D-glucamine, 2 mM $MgCl_2$, 3 mM $BaCl_2$, 10 mM glucose, and 10 mM HEPES (pH 7.4 adjusted with HCl; osmolarity of 320 mosM). In some recordings, *N*-methyl-D-glucamine was replaced with the same concentration of TEA. The pipette internal solution contained 120 mM CsCl, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM EGTA, 4 mM MgATP, and 0.3 mM NaGTP (pH 7.2 adjusted with CsOH; osmolarity of 300 mosM). I_{Ba} was elicited by a series of command potentials from −70 to 50 mV for 150 ms in 10-mV steps (5-s intervals) from a holding potential of −90 mV (12). T-type I_{Ba} was recorded by depolarizing the cells from −90 to −45 mV for 150 ms (12). The steady-state inactivation of VACCs was measured by depolarizing cells to a series of prepulse potentials from −100 to 20 mV for 2500 ms in 10-mV steps (15-s intervals), followed by a command potential to 0 for 150 ms.

To selectively record Kv currents, the extracellular solution contained 150 mM choline chloride, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 1 mM $CdCl_2$, and 10 mM D-glucose (pH 7.4 adjusted with KOH; osmolarity of 320 mosM). The electrode resistance was 1–2 megaohms when filled with a solution containing 120 mM potassium gluconate, 20 mM KCl, 2 mM $MgCl_2$, 10 mM EGTA, 10 mM HEPES, 5 mM Na_2ATP, and 1 mM $CaCl_2$ (pH 7.2 adjusted with KOH; osmolarity of 300 mosM). Kv channel currents were recorded by depolarizing the neurons from −90 to 50 mV for 100 ms, as we described previously (13). Cells in the recording chamber were continuously bathed in the extracellular solution. Each drug solution was delivered to the recording chamber by gravity. Drugs and chemicals were purchased from Sigma, except α-dendrotoxin, phrixotoxin-2, and BDS-I (Alomone Labs, Jerusalem, Israel); CP339818 (Tocris, Ellisville, MO); and 1-methylpiperidin-4-amine (Oakwood Products, West Columbia, SC).

Recording of Excitatory Postsynaptic Currents in the Spinal Cord—Rats were anesthetized with 2–3% isoflurane, and the lumbar segment of the spinal cord was removed through laminectomy. The spinal tissue was immediately placed in ice-cold sucrose artificial cerebrospinal fluid presaturated with 95% O_2 and 5% CO_2. The sucrose artificial cerebrospinal fluid contained 234 mM sucrose, 3.6 mM KCl, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 12.0 mM glucose, and 25.0 mM $NaHCO_3$. The tissue was then placed in a shallow groove formed in a gelatin block and glued onto the stage of a vibratome. Transverse spinal cord slices (400 μm) were cut in the ice-cold sucrose artificial cerebrospinal fluid and preincubated in Krebs solution oxygenated with 95% O_2 and 5% CO_2 at 34 °C for at least 1 h before they were transferred to the recording chamber. The Krebs solution contained 117.0 mM NaCl, 3.6 mM KCl, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 11.0 mM glucose, and 25.0 mM $NaHCO_3$.

Excitatory postsynaptic currents (EPSCs) were recorded using the whole-cell voltage-clamp method, as we described previously (14, 15). The slice was perfused continuously with Krebs solution (5.0 ml/min) at 34 °C maintained by an in-line solution heater and a temperature controller. Lamina II neurons in the spinal slice were identified using a fixed-stage microscope with differential interference contrast/infrared

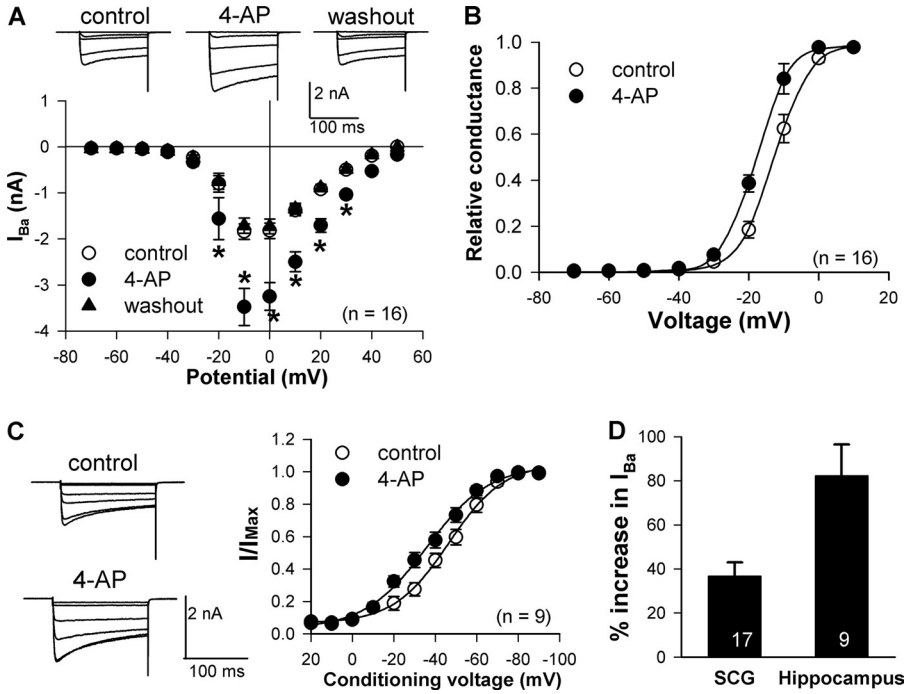

FIGURE 1. **4-AP stimulates HVACC currents in acutely dissociated neurons.** *A*, representative traces and *I-V* curves of I_{Ba} before and during application of 5 mM 4-AP in DRG neurons ($n = 16$). Neurons were voltage-clamped at −90 mV and depolarized from −70 to 50 mV for 150 ms with 10 mV increments. *, $p < 0.05$ compared with corresponding values before 4-AP application. *B*, steady-state activation curves of I_{Ba} before and during 4-AP application in 16 DRG neurons. Before 4-AP application, the $V_{0.5}$ and slope factor were −12.7 ± 0.2 and 5.0 ± 0.2 mV, respectively. During 4-AP application, the $V_{0.5}$ and slope factor were −18.0 ± 0.1 and 4.6 ± 0.1 mV, respectively. *C*, representative current traces and summary data showing steady-state inactivation of I_{Ba} before and during 4-AP application in nine DRG neurons. The $V_{0.5}$ values were −46.5 ± 1.2 and −38.0 ± 1.5 mV ($p < 0.05$) before and during 4-AP application, respectively. The slope factors were −13.3 ± 1.2 and −18.9 ± 2.2 mV ($p < 0.05$) before and during 4-AP application, respectively. Cells were depolarized at a series of prepulse potentials (from −90 to 10 mV, 500 ms) and then depolarized to 0 mV. The normalized conductance (G/G_{max}) was fit to the Boltzmann function. *D*, summary data showing the effects of 4-AP on I_{Ba} in SCG and hippocampal neurons.

illumination. EPSCs were recorded at a holding potential of −60 mV with an electrode (impedance of 5–8 megaohms) filled with the following internal solution: 135.0 mM gluconate, 5.0 mM TEA, 2.0 mM $MgCl_2$, 0.5 mM $CaCl_2$, 5.0 mM HEPES, 5.0 mM EGTA, 5.0 mM MgATP, 0.5 mM NaGTP, and 10 mM QX314. The solution was adjusted to pH 7.2–7.4 with 1 M KOH (osmolarity of 290–300 mosM). EPSCs were evoked by electrical stimulation (0.2 ms, 0.3–0.6 mA, and 0.2 Hz) through a bipolar tungsten electrode placed on the dorsal root zone. Monosynaptic EPSCs were identified on the basis of the constant latency of evoked EPSCs and the absence of conduction failure of evoked EPSCs in response to a 20-Hz electrical stimulation.

Recording of End-plate Potentials—To assess the effect of 4-AP on neuromuscular transmission, we recorded end-plate potentials (EPPs) using a phrenic nerve-diaphragm preparation (16). Rats were anesthetized with isoflurane, and the diaphragm and the attached phrenic nerve were removed rapidly and pinned in a Sylgard-lined 35-mm Petri dish. The phrenic nerve-diaphragm was superfused with oxygenated Ringer's solution containing 116 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM NaH_2PO_4, 23 mM $NaHCO_3$, and 11 mM glucose (pH adjusted to 7.2–7.3), and the solution was continuously gassed with 95% O_2 and 5% CO_2. Muscle contraction was selectively blocked with 2.3 μM μ-conotoxin GIIIB (Bachem, King of Prussia, PA), which preferentially blocks muscle-specific voltage-activated Na^+ channels (17). Intracellular sharp-electrode recording was performed using glass microelectrodes (10–15 megaohms, filled with 3 mM KCl) at 25 °C. Microelectrodes were lowered slowly using a manipulator until EPPs of muscle fibers were recorded. EPPs were evoked with supramaximal stimuli applied to the phrenic nerve via a suction electrode. EPP signals were processed by a Multiclamp 700B amplifier (Molecular Devices, Foster City, CA). The areas under the curve of evoked EPPs were integrated and analyzed before and during drug application.

Data Analysis—The Ca^{2+} and K^+ current data were analyzed using the PulseFit software program (HEKA Instruments). Whole-cell Ca^{2+} current-voltage (*I-V*) relationships for individual neurons were constructed by calculating the mean peak inward current at each test potential. Conductance-voltage (*G-V*) curves were calculated by dividing the current at each potential by the driving force ($V - V_r$), where V is the test potential and V_r is the reversal potential extrapolated from the *I-V* curve. The normalized conductance (G/G_{max}) for *G-V* relationships and inactivation curves were fit with the Boltzmann function: $G/G_{max} = G_{min} + (1/[1 + \exp(V - V_{0.5})/k])$, where G_{min} is the minimal conductance of VACCs, G_{max} is the maximal conductance, $V_{0.5}$ is the voltage for 50% activation or inactivation of VACCs, and k is a voltage-dependent slope factor. The percent augmentations of total I_{Ba} and subtypes of I_{Ba} were calculated as the ratio of 4-AP-augmented I_{Ba} to the total peak I_{Ba} or VACC subtype currents during control, respectively. The effects of 4-AP on EPPs and EPSCs were analyzed using Clampfit (Axon Instruments). Statistical data are presented as means ± S.E. All comparisons between means were tested for significance using Student's paired or unpaired t test or one-way analysis of variance unless indicated otherwise. $p < 0.05$ was considered to be statistically significant.

RESULTS

4-AP Potentiates VACC Currents in Dissociated Neurons—The whole-cell VACC currents in DRG neurons were elicited by a series of depolarizing pulses (from −70 to 50 mV for 150 ms in 5-mV increments) from a holding potential of −90 mV. Bath application of 4-AP (5 mM) caused a large increase in I_{Ba} in all the neurons tested (Fig. 1*A*). The effect of 4-AP was rapid and completely washed out in 3 min. To further characterize the effect of 4-AP on I_{Ba}, we examined the effect of 4-AP on

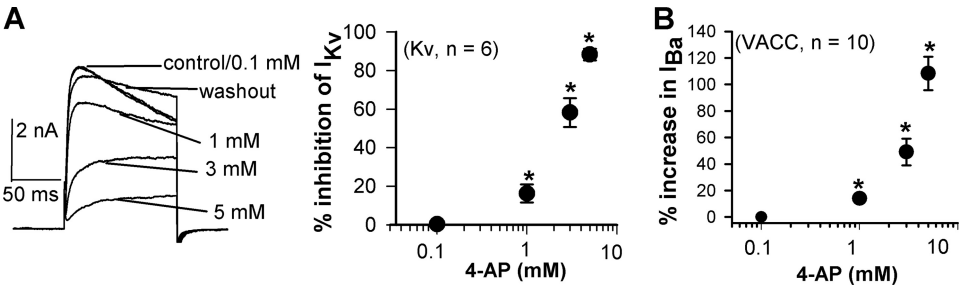

FIGURE 2. **Comparison of the concentration-response effects of 4-AP on Kv channel currents and I_{Ba} in DRG neurons.** *A*, original traces and summary data show the concentration-dependent effects of 4-AP on Kv channel currents in DRG neurons ($n = 6$). *B*, average data show the concentration-dependent effects of 4-AP on I_{Ba} in DRG neurons ($n = 10$). *, $p < 0.05$ compared with the corresponding control.

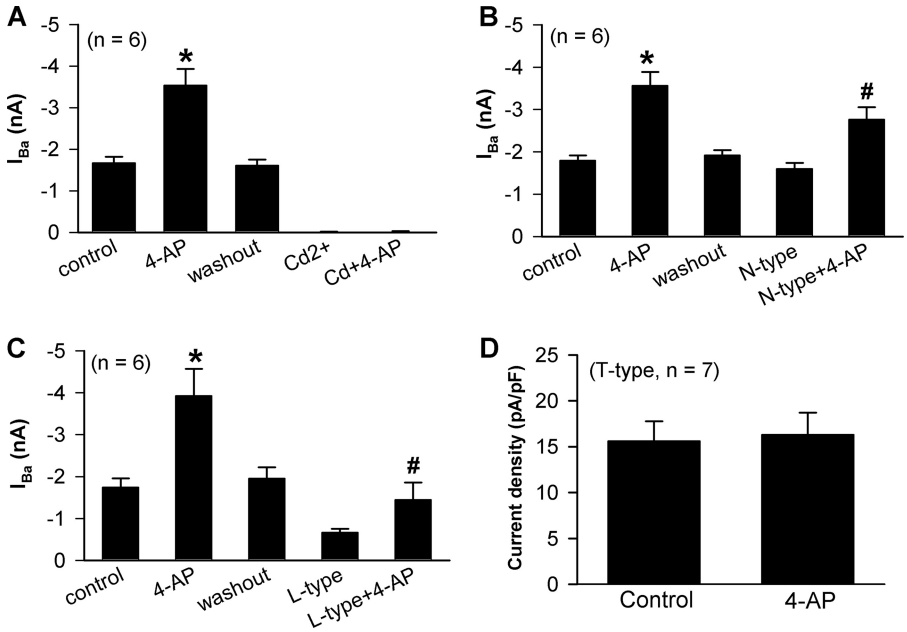

FIGURE 3. **Effects of 4-AP on the subtypes of HVACC currents.** *A*, average data show that cadmium (Cd^{2+}) blocked I_{Ba} and the effect of 5 mM 4-AP on I_{Ba} in DRG neurons. *B*, summary data show that 4-AP increased both the total and N-type Ca^{2+} I_{Ba} in DRG neurons ($n = 6$). *C*, summary data show the effect of 4-AP on the total and L-type I_{Ba} in DRG neurons ($n = 6$). N- and L-types represent pharmacologically isolated N- and L-type Ca^{2+} channel currents. *D*, summary data show a lack of effect of 4-AP on T-type I_{Ba} in DRG neurons ($n = 7$). *, $p < 0.05$ compared with the corresponding control; #, $p < 0.05$ compared with the isolated N- or L-type I_{Ba} amplitude. *pF*, picofarad.

the steady-state activation and inactivation kinetics of I_{Ba}. Although 5 mM 4-AP did not significantly alter the current-voltage relationship, it significantly shifted the steady-state activation to the left ($n = 16$) (Fig. 1*B*). In nine additional neurons, the steady-state inactivation of I_{Ba} was examined through a series of prepulse potentials (−90 to 10 mV for 500 ms), followed by depolarization of the cell to 0 mV for 150 ms. Following application of 5 mM 4-AP, the voltage-dependent steady-state inactivation of I_{Ba} was shifted significantly to the left (*i.e.* more negative potentials) (Fig. 1*C*). Because 4-AP is a well known Kv blocker, we also compared the concentration-effect relationship of 4-AP on I_{Ba} and Kv channel currents in DRG neurons. At the same concentrations (1–5 mM) that blocked Kv channels, 4-AP also significantly potentiated I_{Ba} (Fig. 2). 4-AP had a 50% maximal effect on Kv channels and VACCs at the same concentration (~2.5 mM). When HVACCs were blocked with 100 μM Cd^{2+}, subsequent application of 4-AP failed to increase I_{Ba} ($n = 6$) (Fig. 3*A*).

To determine whether the potentiating effect of 4-AP on VACCs is tissue-specific, we examined the effect of 4-AP on I_{Ba} in dissociated SCG and hippocampal neurons. As in DRG neurons, 5 mM 4-AP substantially increased the amplitude of I_{Ba} in hippocampal neurons ($n = 9$) (Fig. 1*D*). However, the potentiation of I_{Ba} by 5 mM 4-AP in SCG neurons was relatively small ($n = 17$) (Fig. 1*D*) compared with that in DRG and hippocampal neurons.

4-AP Increases N- and L-type, but Not T-type, I_{Ba}—To determine whether 4-AP affects the N- and L-type VACCs, the specific Ca^{2+} channel blockers nimodipine (5 μM, L-type), ω-conotoxin GVIA (2 μM, N-type), ω-agatoxin IVA (100 nM, P/Q-type), and ω-conotoxin MVIIC (500 nM, N- and P/Q-type) were selectively combined to define L- and N-type Ca^{2+} channels (18, 19). In this protocol, the effect of 5 mM 4-AP was initially tested without blockers to determine the augmentation of total I_{Ba}. After the initial effect of 4-AP was washed out, a series of grouped blockers was used to isolate defined subtypes of HVACC currents before the effect of 4-AP was re-examined. 4-AP significantly increased N-type I_{Ba} (isolated using nimodipine and ω-agatoxin IVA; $n = 6$) and L-type I_{Ba} (isolated by co-application of ω-conotoxin GVIA, ω-conotoxin MVIIC, and ω-agatoxin IVA; $n = 6$) (Fig. 3, *B* and *C*). However, 4-AP did not significantly affect T-type I_{Ba} (Fig. 3*D*).

Blocking Voltage-activated K^+ Channels Does Not Mimic or Alter the Effect of 4-AP on I_{Ba}—Because 4-AP is a well known A-type Kv channel blocker, we determined whether the effect of 4-AP on HVACCs is mediated by its effect on Kv channels in DRG neurons. When I_{Ba} was recorded in the *N*-methyl-D-glucamine external solution, bath application of 140 mM TEA, a blocker selectively acting on slow transient outward K^+ currents (20), alone had no significant effect on I_{Ba}. Even in the presence of TEA, 5 mM 4-AP produced a large potentiation of I_{Ba} ($n = 6$) (Fig. 4*A*). 4-AP blocks many Kv subtypes that constitute A-type Kv currents (21–23). In DRG neurons, A-type Kv currents are mediated primarily by Kv1.4, Kv3.4, and Kv4.3 (13, 24). Thus, to test whether blocking Kv channels mimics or alters 4-AP-induced potentiation of I_{Ba} in DRG neurons, we applied 100 nM α-dendrotoxin, a specific Kv1 blocker (25, 26); 1 μM phrixotoxin-2, a specific Kv4.2/Kv4.3 blocker (27); or 1 μM BDS-I (blood-depressing substance-I), a specific Kv3.4 blocker (28). After testing and confirming the initial 4-AP effect on I_{Ba}, these Kv channel blockers were then applied individually to the

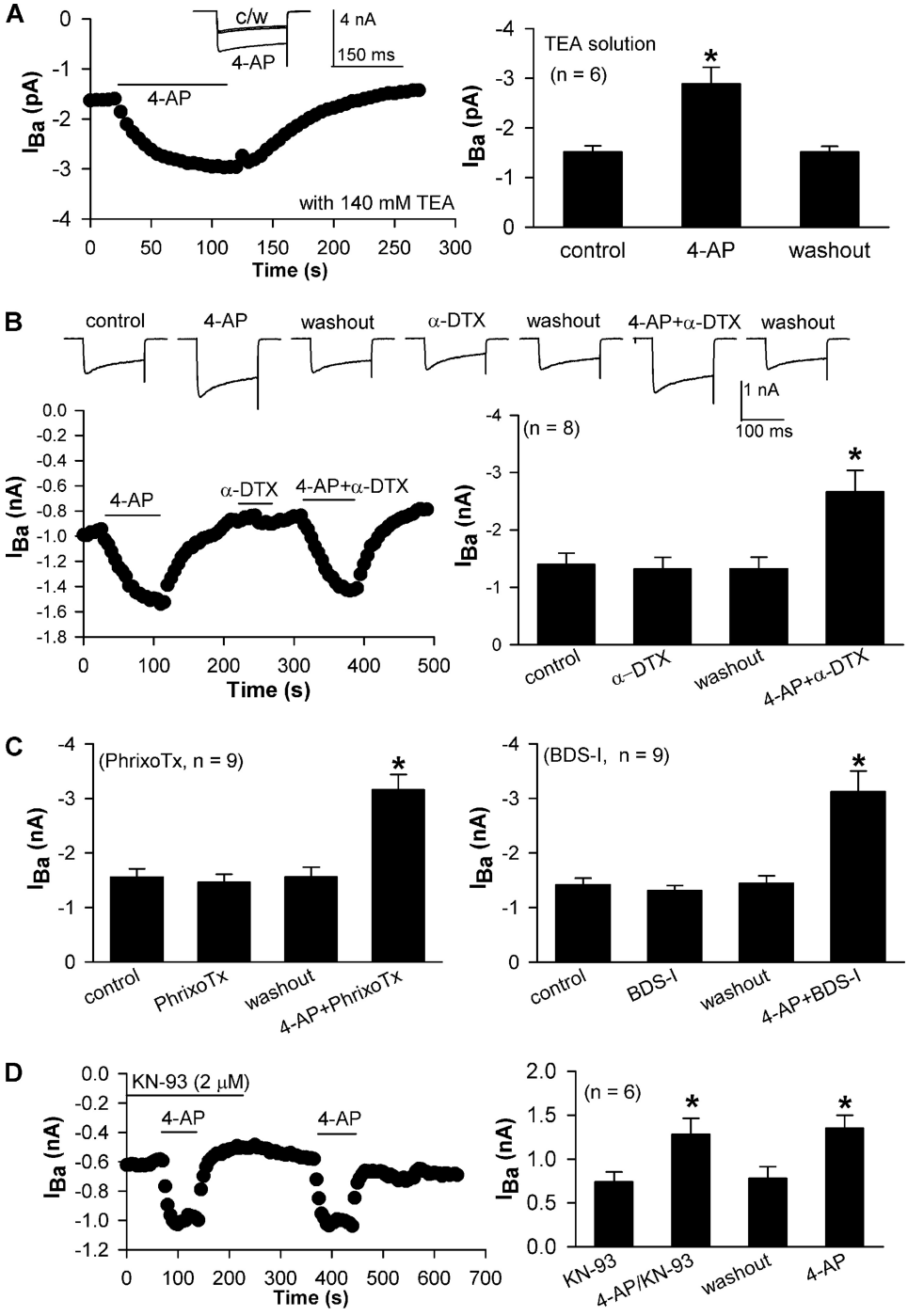

FIGURE 4. **4-AP stimulates HVACCs in DRG neurons independent of Kv channels.** *A*, original traces and summary data show that 140 mM TEA had no effect on I_{Ba} or the effect of 4-AP on I_{Ba}. *B*, representative current traces and summary data show that the effect of 4-AP on I_{Ba} was not altered in the presence of 100 nM α-dendrotoxin (*α-DTX*). *C*, summary data show that 1 μM phrixotoxin (*PhrixoTx*) or 1 μM BDS-I did not mimic or alter the effect of 4-AP on I_{Ba}. *D*, original current traces and summary data show that 2 μM KN-93 did not change 4-AP-induced augmentation of I_{Ba}. *, $p < 0.05$ compared with the corresponding value during the control or washout (*c/w*).

same DRG neurons. None of these A-type Kv blockers alone had any significant effect on I_{Ba}. The potentiating effect of 4-AP on I_{Ba} was not altered by α-dendrotoxin ($n = 8$), phrixotoxin-2 ($n = 9$), or BDS-I ($n = 9$) in any of the DRG neurons tested (Fig. 4, *B* and *C*).

It has been reported that 4-AP may increase Ca^{2+} currents through calmodulin-dependent protein kinase II in cardiomyocytes (29). However, the calmodulin-dependent protein kinase II inhibitor KN-93 used in that study is also a broad-spectrum Kv channel blocker (30). Pretreatment with 2 μM of KN-93 significantly decreased the base-line I_{Ba} in DRG neurons, but it did not significantly alter the potentiating effect of 4-AP on the amplitude of I_{Ba} ($n = 6$, $p > 0.05$) (Fig. 4*D*). When 10 μM KN-93 was included in the pipette solution, moreover, it also failed to alter the effect of 4-AP on I_{Ba} ($n = 7$).

Potentiation of HVACCs by Other Aminopyridines and 4-AP Analogs—We next determined the structure-function relationship of the effects of aminopyridines on HVACCs. The concentration-response effects of 4-AP and other aminopyridines on I_{Ba} in DRG neurons were compared. Different concentrations of aminopyridines were applied in a random order. The cells were depolarized to −10 mV for 150 ms from a holding potential of −90 mV. Although 2-aminopyridine, 3-aminopyridine, and 3,4-DAP all significantly increased the amplitude of I_{Ba}, 4-AP was most efficacious in the potentiating I_{Ba} (Fig. 5, *A* and *B*).

To identify compounds that could be more effective than 4-AP in potentiating HVACCs, we examined the effects of several 4-AP analogs on I_{Ba} in DRG neurons. Among the compounds tested, 4-(aminomethyl)pyridine, 4-(methylamino)pyridine, and 4-di(methylamino)pyridine mimicked the effect of 4-AP on I_{Ba}. At 1 and 3 mM concentrations, 4-di(methylamino)pyridine ($n = 6$), 4-(methylamino)pyridine ($n = 6$), and 4-(aminomethyl)pyridine ($n = 6$) produced equivalent or greater potentiating effects than 4-AP on I_{Ba} (Fig. 5, *A* and *C*). However, the effect of 4-amino-2-methylpyridine on I_{Ba} was less than that of 4-AP. Other pyridine derivatives, including 1-(4-pyridyl)piperazine, cyclohexylamine, and 1-methylpiperidin-4-amine, slightly increased I_{Ba} at a concentration of 5 mM. In addition, aniline or *p*-anisidine slightly inhibited I_{Ba} in DRG neurons.

4-AP Potentiates HVACC Currents through the β Subunit—To further demonstrate a direct effect of 4-AP on HVACCs, we determined the effect of 4-AP on N-type (Cav2.2) channels expressed in HEK293 cells. Cells were transfected with α_1B

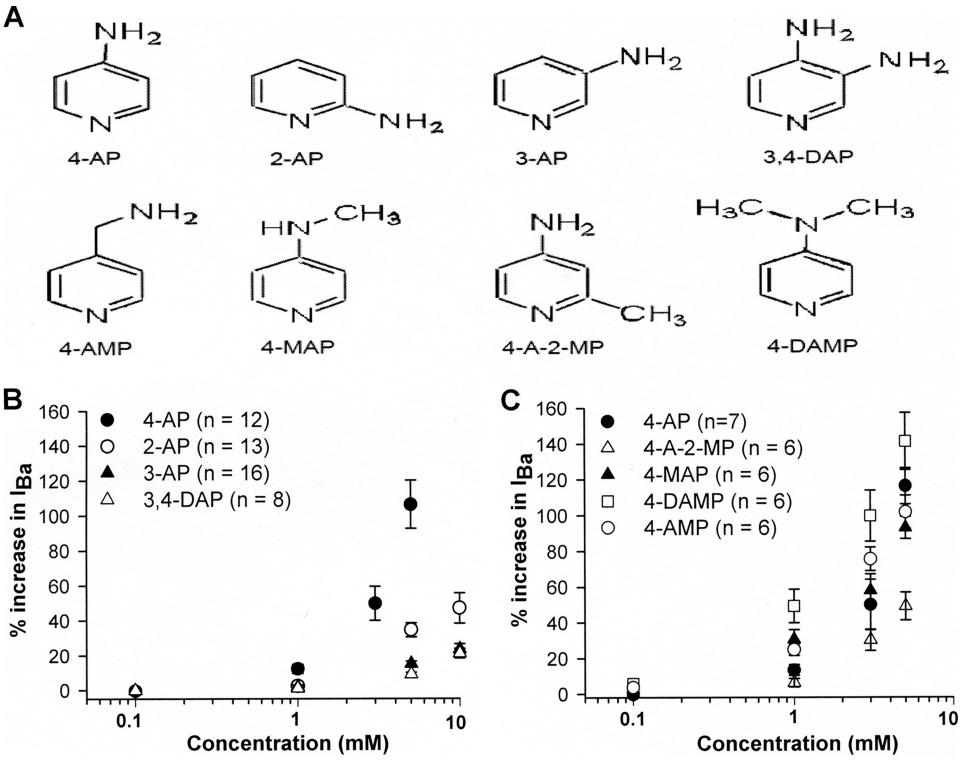

FIGURE 5. **Comparison of the effects of aminopyridines and 4-AP analogs on HVACCs in DRG neurons.** *A*, chemical structure of aminopyridines and 4-AP analogs. *B*, comparison of the concentration-response effects of four aminopyridines on the peak amplitude of I_{Ba}. *C*, comparison of the concentration-response effects of four 4-AP analogs on I_{Ba}. *2-AP*, 2-aminopyridine; *3-AP*, 3-aminopyridine; *4-AMP*, 4-(aminomethyl)pyridine; *4-MAP*, 4-(methylamino)pyridine; *4-A-2-MP*, 4-amino-2-methylpyridine; *4-DAMP*, 4-di(methylamino)pyridine.

alone or in combination with β_3 and $\alpha_2\delta$ subunits. We selected the β_3 subunit because it associates with a majority of N-type HVACCs (31). Application of 5 mM 4-AP significantly increased the amplitude of I_{Ba} in HEK293 cells transfected with α_1B plus β_3 and $\alpha_2\delta$ ($n = 12$) (Fig. 6, *A–C*). However, the amplitude of I_{Ba} in HEK293 cells expressing α_1B alone or α_1B plus $\alpha_2\delta$ was small, and 4-AP only slightly increased I_{Ba} in cells expressing either α_1B alone ($n = 5$) or α_1B plus $\alpha_2\delta$ ($n = 9$) without the β_3 subunit. Strikingly, 4-AP profoundly increased I_{Ba} in cells when β_3 was cotransfected with α_1B or α_1B plus $\alpha_2\delta$ (Fig. 6*C*). Furthermore, 4-AP shifted both steady-state activation and inactivation curves to the left (Fig. 6, *D* and *E*). In contrast, 5 mM 4-AP had no significant effect on T-type I_{Ba} in HEK293 cells transfected with the Cav3.2 subunit ($n = 6$) (Fig. 6*F*).

4-AP Increases Synaptic Transmission in the Spinal Cord Independent of Kv Channels—To determine whether the Kv channels are involved in the potentiating effect of 4-AP on glutamate release from sensory nerve terminals, we examined the effect of 4-AP on glutamatergic EPSCs in the spinal cord evoked from the dorsal root. At concentrations between 0.1 and 2 mM, 4-AP caused concentration-dependent increases in the amplitude of monosynaptic EPSCs (Fig. 7*A*). To block the Kv channels, we used two broad-spectrum A-type Kv channel blockers, α-dendrotoxin (100 nM) for Kv1.1, Kv1.2, Kv1.4, and Kv1.6 (21) and CP339818 (3 μM) for Kv4.2 and Kv4.3 (32). In DRG neurons, 100 nM α-dendrotoxin and 3 μM CP339818 diminished Kv channel currents. Bath application of α-dendrotoxin and CP339818 to the spinal cord slice only slightly increased the amplitude of EPSCs. However, α-dendrotoxin and CP339818 failed to significantly attenuate the potentiating effect of 0.5 mM 4-AP on EPSCs in all of the spinal dorsal neurons examined (Fig. 7*B*). Blocking the glutamate α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors with 20 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) completely abolished EPSCs at the end of the experiment.

4-AP Augments Neuromuscular Transmission Independent of Kv Channels—We investigated whether the potentiating effect of 4-AP on acetylcholine release from motor nerve terminals involves Kv channels. At 0.5 mM, 4-AP substantially prolonged the duration of cholinergic EPPs with a small increase in their amplitude ($n = 10$) (Fig. 8*A*). α-Dendrotoxin (100 nM) and CP339818 (3 μM) were applied to determine the contribution of Kv channels to the effect of 4-AP on EPPs. α-Dendrotoxin and CP339818 alone induced only a small increase in the peak amplitude, but not the duration, of EPPs. Even when applied in the presence of α-dendrotoxin and CP339818, 0.5 mM 4-AP still profoundly increased the duration and amplitude of EPPs ($n = 11$). The potentiating effect of 4-AP on the size of EPPs, quantified using the area under the curve, was not significantly altered by α-dendrotoxin and CP339818 (Fig. 8*B*). Bath application of the nicotinic acetylcholine receptor antagonist D-tubocurarine (5 μM) blocked EPPs at the end of the experiments.

DISCUSSION

HVACCs at the presynaptic terminals are essential for synaptic and neuromuscular transmission. The potentiating effects of aminopyridines on neurotransmitter release are believed to result from blocking Kv channels, which subsequently leads to prolongation of the action potential, followed by activation of HVACCs. However, findings from our study directly challenge this conventional view by demonstrating that 4-AP and many of its analogs directly stimulate HVACCs in dissociated neurons. We found that 4-AP, at the same concentration that blocked Kv channels, significantly increased the amplitude of I_{Ba} in neurons isolated from the DRG, SCG, and hippocampus. We also found that 4-AP significantly augmented N- and L-type, but not T-type, VACCs. 4-AP-induced augmentation of I_{Ba} is likely mediated by HVACCs because 4-AP failed to produce any effect on I_{Ba} when HVACCs were blocked with Cd^{2+}. Under our recording conditions, K^+ was removed from both internal and external solutions. Thus, the potentiating effect of 4-AP on HVACCs is not associated with K^+ ions permeating through Kv channels. Furthermore, TEA, a

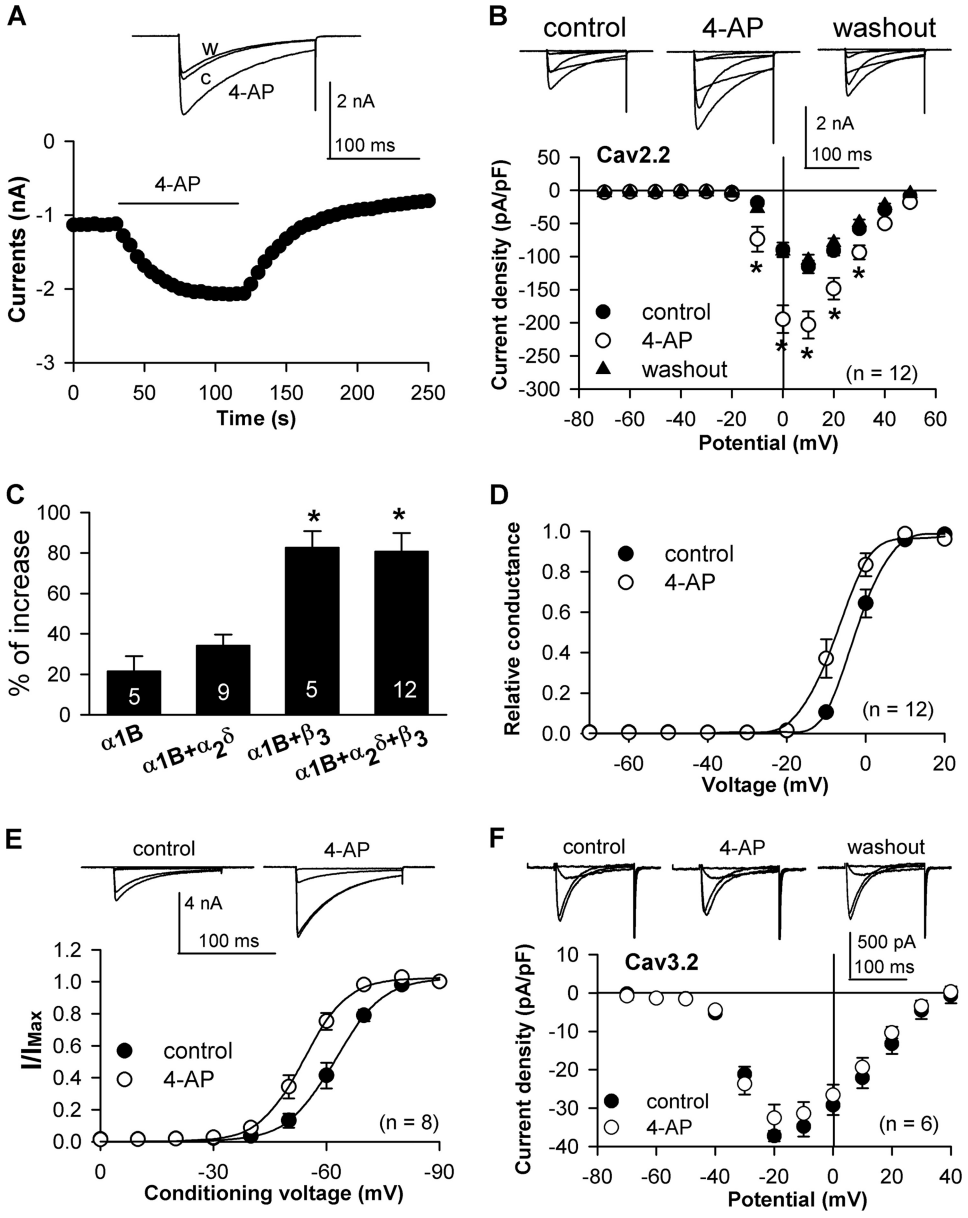

FIGURE 6. **4-AP stimulates N-type (Cav2. 2) HVACCs through the β_3 subunit expressed in HEK293 cells.** *A*, raw current traces and time course showing that 5 mM 4-AP increased N-type I_{Ba}. *B*, current traces and *I-V* curves showing that 4-AP increased N-type I_{Ba}. Cells were voltage-clamped at −90 mV and depolarized from −70 to 50 mV for 150 ms with 10-mV increments. *, $p < 0.05$ compared with corresponding values before 4-AP application. *C*, comparison of the effects of 4-AP on N-type I_{Ba} reconstituted with different combinations of N-type Ca^{2+} channel subunits. *, $p < 0.05$ compared with the effect of 4-AP on I_{Ba} without the β_3 subunit. *D*, steady-state activation curves of N-type I_{Ba} (α_1B + β_3 + $\alpha_2\delta$) before and during 4-AP application in 12 HEK293 cells. Before 4-AP application, the $V_{0.5}$ and slope factor were −2.3 ± 0.1 and 3.6 ± 0.1 mV, respectively. During 4-AP application, the $V_{0.5}$ and slope factor were −7.8 ± 0.3 and 4.0 ± 0.3 mV, respectively. Neurons were voltage-clamped at −90 mV and depolarized from −70 to 20 mV for 150 ms with 10-mV increments. *E*, original current traces and summary data showing that 4-AP shifted the steady-state inactivation curve of N-type I_{Ba} (α_1B + β_3 + $\alpha_2\delta$) toward the left ($n = 8$). The $V_{0.5}$ and slope factor before 4-AP application were −62.3 ± 0.4 and −6.3 ± 0.3 mV, respectively. After 4-AP, the $V_{0.5}$ and slope factor were −54.0 ± 0.3 and −5.8 ± 0.3 mV, respectively. *F*, current traces and *I-V* curves showing the lack of effect of 4-AP on T-type (Cav3.2) Ca^{2+} channels expressed in HEK293 cells. *w*, washout; *c*, control; *pF*, picofarad.

Kv channel blocker acting mainly on slow transient outward K^+ currents (20), did not mimic or attenuate the potentiating effect of 4-AP on HVACC currents. We found that three highly specific A-type Kv channel blockers, α-dendrotoxin (Kv1), BDS-I (Kv3.4), and phrixotoxin-2 (Kv4.2 and Kv4.3), all failed to mimic or alter the effect of 4-AP on HVACCs. Collectively, our data provide strong evidence that 4-AP potentiates HVACCs independent of Kv channels in neurons.

By determining the structure-function relationship of aminopyridines on I_{Ba}, we found that 4-AP (the amine at position 4 of the pyridine ring) is the aminopyridine that is most effective in stimulating HVACCs. Although 3,4-DAP shows a greater potency than 4-AP in blocking Kv channels (33), 4-AP seems to be superior to 3,4-DAP in the treatment of motor dysfunction in patients with multiple sclerosis (34). Consistent with this clinical observation, we found that 4-AP produced a greater effect than 3,4-DAP on HVACCs. By testing the effects of 4-AP analogs on I_{Ba}, we demonstrated that 4-(aminomethyl)pyridine, 4-(methylamino)pyridine, and 4-di(methylamino)pyridine potentiated HVACCs with a similar or greater potency than 4-AP. Therefore, our findings suggest that 4-AP can be derivatized to yield compounds more effective in stimulating HVACCs. 4-AP analogs such as 4-di(methylamino)pyridine may be more efficacious than 4-AP in the treatment of neuromuscular dysfunctions.

To further identify the subunit(s) involved in the potentiating effect of 4-AP on HVACCs, we determined the effect of 4-AP on N-type I_{Ba} reconstituted by expressing α_1B, β_3, and $\alpha_2\delta$ subunits in HEK293 cells. We found that 4-AP similarly produced a profound effect on the current amplitude of N-type, but not low voltage-activated T-type (Cav3.2), VACCs. The major pharmacological and functional properties of the N-type HVACCs are determined by the pore-forming α_1B subunit (35, 36). In our study, 4-AP had only a small effect on I_{Ba} when α_1B was expressed alone or with the $\alpha_2\delta$ subunit. Strikingly, 4-AP profoundly potentiated I_{Ba} when the β_3 subunit was coexpressed with α_1B or α_1B plus $\alpha_2\delta$. Therefore, the intracellular β_3 subunit is the most important site of the HVACC-potentiating activity of 4-AP. The function of T-type VACCs does not require any β subunit, which explains the lack of effect of 4-AP on T-type I_{Ba} in dissociated neurons and HEK293 cells. The β subunit of HVACCs affects the channel function mainly

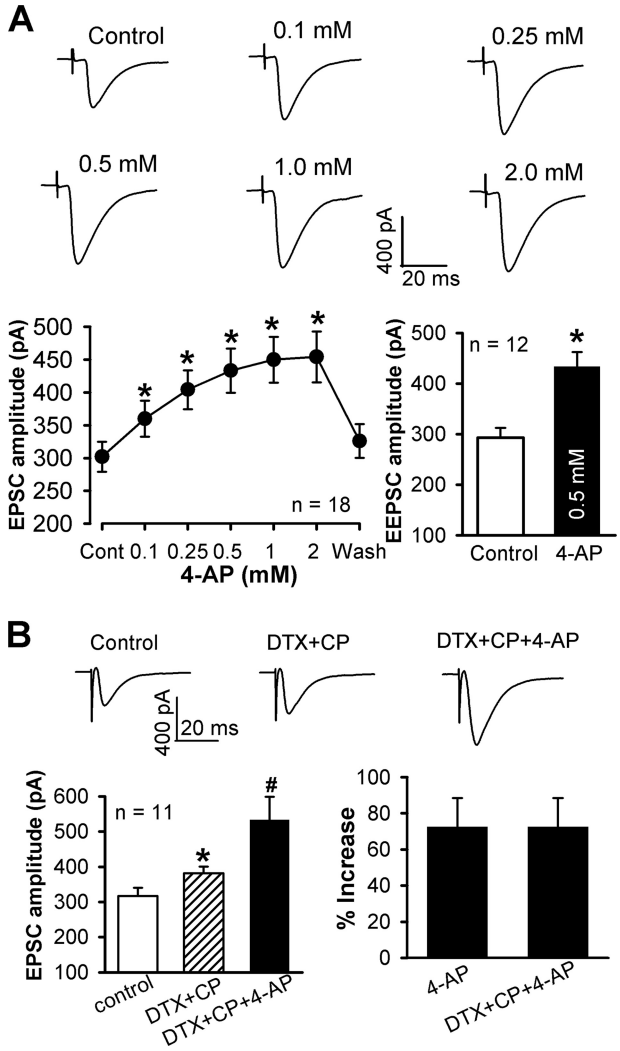

FIGURE 7. **4-AP increases glutamate release from primary afferent terminals in the spinal cord independent of Kv channels.** *A*, original traces and summary data show the concentration-dependent effect of 4-AP on evoked monosynaptic glutamatergic EPSCs of spinal dorsal horn neurons. *B*, representative recordings and summary data show that 100 nM α-dendrotoxin (*DTX*) and 3 μM CP339818 (*CP*) had no significant effect on 4-AP-induced increases in the amplitude of EPSCs of dorsal horn neurons. *, $p < 0.05$ compared with the control (*Cont*); #, $p < 0.05$ compared with the effect of α-dendrotoxin and CP339818 alone.

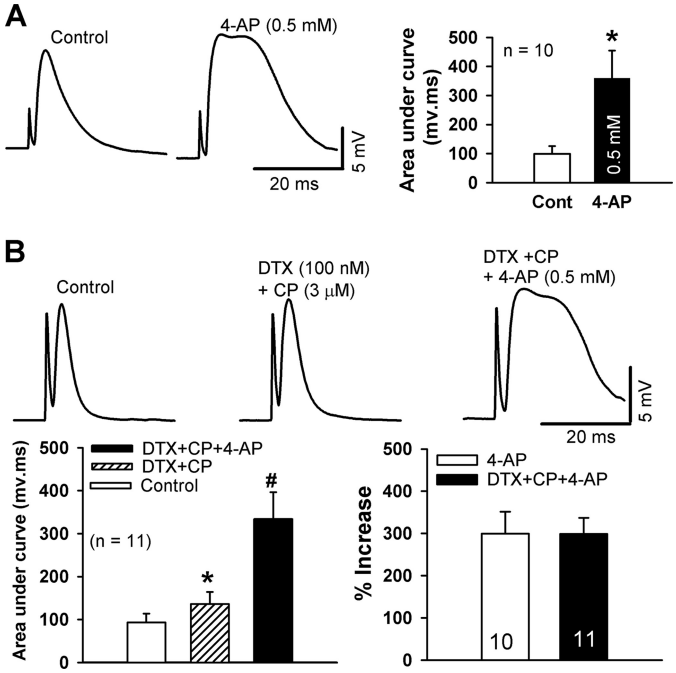

FIGURE 8. **4-AP potentiates acetylcholine release from motor nerve terminals in the phrenic nerve-diaphragm independent of Kv channels.** *A*, original traces and summary data show the effect of 0.5 mM 4-AP on evoked cholinergic EPPs. *B*, representative recordings and summary data show that 100 nM α-dendrotoxin (*DTX*) and 3 μM CP339818 (*CP*) had no significant effect on 4-AP-induced potentiation of EPPs. *, $p < 0.05$ compared with the control (*Cont*); #, $p < 0.05$ compared with the effect of α-dendrotoxin and CP339818 alone.

by increasing the current amplitude and by hyperpolarizing the voltage dependence of activation. The basis for these effects of the β subunit is likely an increased number of channels expressed on the plasma membrane (37). We observed that 4-AP induced a hyperpolarizing shift in steady-state activation and inactivation, suggesting that HVACCs may recover more readily from inactivation and can be opened more effectively by the depolarization in the presence of 4-AP. Hence, the effects of 4-AP on the amplitude and activation and inactivation kinetics of I_{Ba} in DRG neurons and HEK293 cells consistently suggest an important role for the β subunit in 4-AP-induced stimulation of HVACCs.

Four β subunits of HVACCs have been identified: β_1, β_2, β_3, and β_4. The β_1 subunit appears to be the only β subunit expressed in skeletal muscle (38). In contrast, only the β_2 subunit is present in rat heart (39). The β_3 subunit is strongly expressed in smooth muscle and brain, and the β_4 subunit is the predominant subunit in the cerebellum (39). The presence of the β_3 subunit in sensory neurons is suggested by the finding that knock-out of the β_3 subunit in mice impairs sensory processing (40). We found that 4-AP had a profound effect on HVACCs in DRG and hippocampus neurons. On the other hand, 4-AP only slightly increased I_{Ba} in SCG neurons, possibly because of the limited expression of the β_3 subunit in the sympathetic neurons. Consistent with our findings, the side effects of 4-AP are generally limited to the gastrointestinal tract and central nervous system with little effect on the autonomic nervous system in humans (41, 42). Thus, the magnitude of the potentiating effect of 4-AP on HVACCs varies among different populations of neurons, likely because of heterogeneous distribution of the β_3 subunit. Further studies are warranted to determine the potential roles of other β subunits in the effect of 4-AP on different subtypes of HVACCs.

Because the effects of 4-AP on HVACCs occur at mM concentrations, it could be argued that this mechanism of action may not be pertinent to its effect on neurotransmitter release and neuromuscular function that emerges at μM concentrations. Notably, the blocking effect of 4-AP on Kv channels also requires mM concentrations, as we found in dissociated neurons. To determine whether Kv channels play a role in the effect of 4-AP on neurotransmitter release at the nerve terminals, we recorded glutamatergic EPSCs evoked from sensory nerve terminals in the spinal cord and cholinergic EPPs elicited by stimulation of the phrenic (motor) nerve. In both preparations, although blocking Kv channels slightly increased the amplitude

of EPSCs and EPPs, it did not mimic or alter the large potentiating effects of 4-AP on EPSCs and EPPs. These findings provide further evidence that 4-AP can promote neurotransmitter release at the presynaptic terminals independent of its effect on Kv channels. Although it is not clear why 4-AP stimulates the nerve terminals at a much lower concentration than is required to potentiate HVACCs at the soma, it is possible that there is a strong expression of the β_3 subunit and/or a very close association between HVACCs and vesicles at the nerve terminals (43, 44).

In summary, our study provides novel evidence that 4-AP and its analogs can directly stimulate HVACCs in neurons independent of Kv channels. Furthermore, the intracellular β subunit is the critical site where 4-AP acts to increase the function of HVACCs. We also demonstrated that 4-AP enhances synaptic and neuromuscular transmission independent of Kv channels. This new information is essential to our understanding of the molecular mechanism of the therapeutic actions of aminopyridines. Identification of the β_3 subunit as the target for the effect of 4-AP on HVACCs has important therapeutic implications because this subunit could be targeted for further development of more efficacious drugs to treat neuromuscular dysfunction. Our findings directly challenge the long-held view that aminopyridines such as 4-AP and 3,4-DAP produce their effects on neurotransmitter release and neuromuscular function through blocking Kv channels. 4-AP is still being widely used as a specific Kv channel blocker to increase intracellular Ca^{2+} and neurotransmitter release. These 4-AP actions should be reinterpreted on the basis of this new information.

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